Euphytica **127:** 353–365, 2002.

PCR-based markers to differentiate the mitochondrial genomes of petaloid and male fertile carrot (*Daucus carota* L.)

Inga C. Bach[1], Annette Olesen[1] & Philipp W. Simon[2,*]
[1]*Department of Agricultural Sciences, Plant Breeding and Crop Science, The Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark;* [2]*United States Department of Agriculture, Agricultural Research Service, Vegetable Crops Research Unit, Department of Horticulture, University of Wisconsin-Madison, 1575 Linden Drive, Madison, Wisconsin, USA; (*author for correspondence, e-mail: psimon@facstaff.wisc.edu)*

Received 24 July 2001; accepted 12 February 2002

Key words: Daucus carota, mitochondria, CMS, petaloidy, PCR-markers

Summary

Cytoplasmic male sterility (CMS) is essential for the development of highly adapted and uniform hybrid varieties of carrot and other crops. The most widely used type of CMS in carrot is petaloidy, in which the stamens are replaced by petals or bract-like structures. We have developed a series of mitochondria-specific PCR markers to distinguish cytoplasms inducing petaloidy (Sp) and male-fertility (N). The markers target the *atp1*, *atp6*, *atp9*, *orfB* (*atp8*), *nad6* and *cob* loci from the mitochondrial genomes of a diverse collection of male fertile and petaloid carrots. We report 14 primer pairs that amplify marker fragments from either Sp or N cytoplasms and three primer pairs that amplify fragments with length polymorphism. The amplification products span sites of insertions, deletions or recombinations adjacent to or within the coding regions of the targeted genes. The markers reported here are useful tools to identify the type of cytoplasm in cultivated carrot and to evaluate variation in the mitochondrial genomes within the genus *Daucus*.

Introduction

Cytoplasmic male sterility (CMS) in higher plants is a maternally inherited trait that has been associated with modifications of the mitochondrial genome or with incompatibility between nuclear and mitochondrial genomes. Several cytoplasms that induce CMS in carrot have been identified and evaluated for utilization in breeding programs. Many of these induce 'brown anther' (Sa) male sterility in which the anthers are formed but shriveled. Sa CMS cytoplasms have been isolated from several open pollinated varieties as well as from wild populations (Welch & Grimball, 1947; Banga et al., 1964; Ronfort et al., 1995). A second CMS type in carrot is 'petaloid' (Sp), in which the stamens are replaced by petals or bract-like structures. This type of cytoplasm has occurred spontaneously in feral populations of (*Daucus carota*) ssp. *carota* from North America and Sweden and in a *D. c. maritimus* accession from Portugal (Bowes & Wolyn, 1998; McCollum, 1966; Morelock et al., 1996; Nothnagel et al., 2000; Thompson, 1961). Additional CMS phenotypes have been observed in carrot lines containing cytoplasms from *Daucus carota* subspecies (Linke et al., 1999) but these cytoplasms have not yet been incorporated in to cultivated carrots for hybrid breeding.

Brown anther CMS was the first type used for developing hybrid carrots, but the majority of current hybrid carrot varieties utilize petaloid cytoplasms derived from two different sources of Sp cytoplasm, designated 'Cornell' (SpC; Thompson, 1961) and 'Wisconsin Wild' (SpW; Morelock et al., 1996). The SpC and SpW cytoplasms were found in Massachusetts and Wisconsin, respectively. Comparison of SpC, SpW and Canadian Sp cytoplasms with the cytoplasms of maintainer lines (N cytoplasms) using mitochondrial restriction length fragment polymorphisms

(RFLPs) revealed that the Sp cytoplasms were very similar to each other and clearly distinct from N cytoplasms, which were found to be substantially more variable (Bowes & Wolyn, 1998; Pingitore et al., 1989; Scheike et al., 1992; Steinborn et al., 1992). A chloroplast RFLP marker has been identified among N cytoplasms of cultivated carrot (Vivek et al., 1999), but chloroplast markers that distinguish N from Sa and Sp cytoplasms have not been found (Scheike et al., 1992; Vivek & Simon, 1999).

Molecular markers that can predict the CMS status at an early developmental stage will be valuable tools in carrot breeding and seed production programs, as well as for basic studies of male sterility. Recently, six mitochondria-specific Sequence Tagged Site (STS) primer pairs derived from Randomly Amplified Polymorphic DNA (RAPD) markers were reported to distinguish SpC and SpW cytoplasms from a collection of three male fertile inbred carrot lines and five open pollinated varieties (Nakajima et al., 1999). However, only two of these primer pairs amplified fragments that were consistently associated with either Sp or N cytoplasms. Because of the high degree of variation among mitochondrial genomes of fertile carrots and the dynamic nature of the mitochondrial genome of higher plants in general, several markers may be required to determine whether an uncharacterized cytoplasm confers petaloidy. Furthermore, a range of mitochondria-specific markers will be useful to characterize the mitochondrial genomes of putative cybrids resulting from protoplast fusions, and to characterize a wide range of wild and cultivated carrot germplasm.

In this study, we utilized a very diverse collection of commercial petaloid carrot inbreds and we have developed a series of mitochondria-specific PCR markers that distinguish the petaloidy-inducing Sp cytoplasms from N cytoplasms. The markers are anchored in conserved mitochondrial coding regions or in the immediate flanking regions. Eleven of the 17 primer pairs reported here amplify marker fragments that are consistently associated with either Sp or N cytoplasms.

Materials and methods

Plant material

We evaluated a collection of six USDA male fertile maintainer (N cytoplasm) B line inbreds and their petaloid (SpC or SpW cytoplasm) male sterile near-isogenic A line counterparts (Table 1). In addition to these seven A-B pairs we examined the commercial B line K831B and the petaloid A line K826A from L. Danefeld A/S as well as the commercial hybrid 'Savory' (SunSeeds) which is also in petaloid cytoplasm. The purpose for selecting these nine petaloid CMS and eight male fertile lines was to include a diverse array of commercial storage root types as well as both SpC and SpW petaloidy. These lines represent the breadth of diversity among a subset of 30 petaloid inbreds or hybrids preliminarily evaluated for several mitochondrial markers. Plants were grown in the green house and total DNA was extracted from leaf tissue by the CTAB-method according to Murray & Thompson (1980).

Preparation of plasmid libraries with mtDNA

Suspension cultures of the petaloid line K826A and the male fertile line K831B were established from individual seedlings and cultivated as described in Bach et al. (1997). Mitochondria were isolated from suspension cells by differential centrifugation and incubated with DNaseI according to Schuster et al. (1988). DNA was isolated from the mitochondrial pellet, digested with *Hin*dIII, *Sal*I or *Xba*I and ligated into the pBluescript® II SK+ vector (Stratagene) and transformed into *E. coli* strain DH5-alpha using standard techniques (Sambrook et al., 1989).

Probe preparation and screening of libraries

Plasmids isolated from colonies from each library were dot-blotted onto Hybond N+ nylon membranes (Amersham) according to the manufacturer's recommendations and the libraries were screened by hybridizing to the dot-blots with mitochondrial probes. Probes for initial screening of the libraries were prepared by labeling of PCR amplification products. The hybridizations were either performed according to the protocol for the ECL Nucleic Acid Labeling and Detection System (Amersham) or the probes were radiolabeled with the DECAprime II™ kit (Ambion Inc., Austin, TX) and the hybridizations were performed using standard methods according to Sambrook et al (1989). The primer sequences for the initial library screenings were based on the sequences of the mitochondrial genes *atp1* (5′-CTTGAGAATGAGAATGTCGG and 5′-TTAATTGTCCCACAGTCGAG), *atp6* (5′-TCCATCTTTGTTCATGCTGC and 5′-CTAAAAAAGGTGCTAACGGC), *atp9*

Table 1. Carrot varieties and breeding lines analyzed with the mitochondria specific markers. The marker fragments A/321-D/1346 are anchored in the *atp1* loci (Figure 1), fragments E/1100 and F/373 in the *atp6* loci (Figure 2); G/427 and G/469 in the *atp9* loci (Figure 3), fragments H/1508-K/751 in the *atp8* loci (Figure 4), fragments L/422-O/790 in the *nad6* loci (Figure 5) and the marker fragments P/1229 and Q/672 are anchored in the *cob-orf25* loci (Figure 6). The Sp1a/1503, Sp1b/1961 and Sp2/1747 fragments are specific for each of three 3′-configurations of *atp8* in petaloid carrot lines (Figure 4). I = Imperator root type, N = Nantes, I-N = Imperator-Nantes, and D-Danvers-Chantenay. 0 = absence, 1 = presence of a clear band, 9 = presence of a faint band. * Indicates deviation from the marker patterns that was predicted based on sequence analysis of the inbred lines K826A and K831B. ** Indicates a 2010 bp amplification product that could not be easily distinguished from Sp1b/1961 on agarose gels. *** Marker summary indicates number observed out of 8 maximum for N cytoplasm, or 9 maximum for Sp cytoplasm

Line/ variety	Root type	Cyto-plasm	*atp1*					*atp6*		*atp9*		*atp8*						*nad6*				*cob*		*atp8*		
			A/ 321	A/ 392	B/ 1632	C/ 1608	D/ 1346	E/ 1100	F/ 373	G/ 427	G/ 469	H/ 1508	I/ 401	I/ 435	I/ 214	J/ 367	K/ 751	L/ 422	M/ 451	N/ 795	O/ 790	P/ 1229	Q/ 672	Sp1a/ 1503	Sp1b/ 1961	Sp2/ 1747
B493A	I	SpC	1	0	1	0	0	1	0	1	0	1	1	0	0	0*	0	1	1	0	9*	1	0	1	1	1
B493B	I	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1**	0
B2566A	N	SpW	1	0	1	0	0	1	0	0*	0	1	1	0	0	0*	0	1	1	0	0	1	0	9*	1	0*
B2566B	N	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1**	0
B3475A	I-N	SpC	1	0	1	0	0	1	0	1	0	1	1	0	0	0*	0	1	1	0	0	1	0	1	1	1
B3475B	I-N	N	0	1	0	1	0*	0	1	0	1	0	0	0*	1*	0	1	0	1*	0*	0*	0	0*	0	0*	0
B6333A	I	SpW	1	0	1	0	0	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1	1
B6333B	I	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1**	0
B7241A	I	SpC	1	0	1	0	0	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1	1
B7241B	I	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1**	0
B9304A	D-C	SpC	1	0	1	0	0	1	0	1	0	1	1	0	0	0*	0	1	1	0	0	1	0	1	1	1
B9304B	D-C	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	0*	0
K803A	I	SpC	1	0	1	0	1*	1	0	1	0	1	0*	0	0	1	0	1	1	0	0	1	0	0*	0*	1
K803B	I	N	0	1	0	1	1	0	1	0	1	0	0	0*	1*	0	1	0	1*	0*	0*	0	0*	0	0*	0
K826A	I	SpC	1	0	1	0	0	1	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	1	1	1
K831B	I	N	0	1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1**	0
Savory	I-N	SpC	1	0	1	0	0	1	0	0*	0	1	1	0	0	0*	0	1	1	0	0	1	0	1	1	9*
Marker summary***	N	0	8	0	8	7	0	8	0	8	0	0	6	2	0	8	0	2	6	6	0	6	0	5	0	
	Sp	9	0	9	0	1	9	0	7	0	9	8	0	0	4	0	9	9	0	1	9	0	8	8	8	

(5′-CAACCCGAGATGTTAGAAGG and 5′-TTGCTTTATGAGACTGAATGG) and *cob* (5′-ATTACAAGCTTAGCTAGCGC and 5′-GCTTTGTCAGGTATACTACG) from *Arabidopsis thaliana* (Unseld et al., 1997). The *atp1*, *atp9* and *cob* fragments were amplified from the carrot lines K826A and K831B. The *atp6* fragment was amplified from *A. thaliana*, since mismatches of the *A. thaliana*-derived primers with the mitochondrial genome of carrot prevented amplification of the corresponding *atp6* fragment from carrot.

Inverse PCR, long range PCR, sequencing and amplification of marker fragments

Mitochondrial DNA regions of interest were cloned either by screening of the mtDNA libraries or by inverse PCR. Inverse PCR was performed with TaKaRa Ex Taq™ (PanVera Corp., Madison, WI) using digested and religated total DNA or mtDNA as the template. The standard buffer and dNTPs supplied by the manufacturer were used and the PCR conditions were: 94 °C for 2 min (1 cycle) followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and 30 sec and one cycle of 72 °C for 7 min.

To determine corresponding 5′- and 3′-regions in cases where contigs could not be confirmed by alignment of overlapping clones, long range PCR was performed with TaKaRa Ex Taq™ in LA-buffer according to the manufacturer's recommendations. The PCR conditions for long range PCR were: 94 °C for 2 min (1 cycle) followed by 30 cycles at 98 °C for 20 sec, 55 °C for 30 sec, 68 °C for 10 min and one cycle of 72 °C for 7 min.

The ABI Prism® BigDye™ Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA) was used for sequencing according to the manufacturer's recommendations. Sequencing gels were run by the University of Wisconsin Biotechnology Center (Madison, WI). Searches of databases were performed using BLASTN (Altschul et al., 1997).

Amplitaq® polymerase (PE Biosystems, Foster City, CA) with supplied reaction buffer was used to amplify the marker fragments in 20 μl reaction mixtures containing 0.4 μg/ml DNA, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.1 mm each of dATP, dCTP, dGTP and dTTP and 0.5 units of Amplitaq® polymerase. The PCR conditions were the same as for inverse PCR with the following exceptions. The annealing temperature was increased to 56 °C for the markers N/795, and O/790 and to 58 °C for Sp1a/1503. The sequences of the primers used for amplification of the marker fragments are listed in Table 2.

Nomenclature

The male sterile petaloid cytoplasms 'Cornell' and 'Wisconsin Wild' were designated SpC and SpW, respectively. Petaloid cytoplasms more generally were designated Sp. Fertility inducing cytoplasms were designated N. The mitochondrial genes were named according to the type of cytoplasm from which they were isolated, e.g. *atp1-Sp*. If more than one copy was isolated from a single cytoplasm, the copies were numbered, e.g. *atp1-N1* and *atp1-N2*. Primers annealing to conserved coding regions were designated according to the corresponding gene and the orientation of the primer ('u' for 5′ and 'd' for 3′), e.g. nad6-u1 and nad6-d1, which anneal to the *nad6* coding region and prime towards the 5′ and 3′-flanking region, respectively. Primers that anneal to intergenic regions of the carrot mtDNA were designated cmt followed by an arbitrary number.

Results

Development of markers and amplification of marker fragments

All markers developed in this work were initially based on differences in the mitochondrial genomes of the sterility maintainer line K831B and the petaloid line K826A bearing the SpC cytoplasm. The markers reflect rearrangements in the vicinity of the conserved coding regions of the mitochondrial genes *atp1*, *atp6*, *orfB* (*atp8*, Gray et al., 1998), *atp9*, *cob* and *nad6*. Three primer pairs spanned insertion-deletions and amplified fragments from both K826A and K831B and 14 primer pairs spanned break points and amplified fragments from either K826A or K831B. In addition, three primer pairs distinguished individual copies of *atp8* genes in Sp cytoplasms.

A direct repeat upstream from the atp1 *coding regions in K831B and variable 3′-flanking regions*

Southern hybridization to *Bam*HI digested total DNA from the petaloid line K826A and the maintainer line K831B indicated that only one *atp1* gene (*atp1-Sp*) was present in K826A while K831B contained two copies of *atp1* (*atp1-N1* and *atp1-N2*, data not shown). These three genes and their flanking regions were

Table 2. Carrot mitochondria-specific markers, primers and annealing sites. Locations of markers are indicated in Figures 1–6. Designation of 5′ or 3′ annealing sites refers to the flanking regions of an ORF to which the primer anneals

Marker	Primer	Sequence	Annealing site
A/321, A/329	cmt-1	5′-GCAAGAAGGAAAGCTGTTAGAG	*atp1* 5′
A/321, A/329	cmt-2	5′-GGTATCCCTCTTCTGTTTCGG	*atp1* 5′
B/1632, C/1608, D/1346	atp1-d1	5′-CGCGTTGGGAGTACCTATTGA	*atp1*
B/1632	cmt-3	5′-GAGGAATACGACGTGAGACAACA	*atp1-Sp* 3′
C/1608	cmt-4	5′-TCCCGCAGGTCGTCAACAG	*atp1-N1* 3′
D/1346	cmt-5	5′-ATCCCCCTCCGCCCCCAGTA	*atp1-N2*
E/1100	cmt-6	5′-GGCAACAGCTGACCACGGTTC	*atp6-Sp1* 5′
E/1100	cmt-7	5′-ATTGCGGCCTTCGCTCCTCGC	*atp6* 3′
F/373	cmt-8	5′-CTACTCAATTTGCACCTGAAC	*atp6-N1* 5′
F/373	atp6-u1	5′-TTTGTGAATGAGAAATACAAG	*atp6*
G/427, G/469	atp9-d1	5′-GAAGGTGCAAAATCAATAGG	*atp9*
G/427, G/469	cmt-9	5′-TACATGGACTTTAAATTGACTTCT	*atp9* 3′
H/1508	cmt-10	5′-GGCCCAGTCAGCAAGCCAGGC	*atp8-Sp* 5′
H/1508	atp8-u1	5′-GTGAATTTATCCAGTTGAGGC	*atp8*
I/214, I/401, I/435	atp8-d2	5′-GTCCTCATATAGCACTTCTTCC	*atp8*
I/214, I/401, I/435	cmt-12	5′ CTGTATTCGTGGGGGGTCGC	*atp8* 3′
J/367	cmt-11	5′-TCACTACTCGTACAGGAAGGACTCTC	*atp8* 5′
J/367	cmt-14	5′-CCTACTTAGTGAAAAAAAAAATTGGAAAAAG	*atp8-Sp2*
K/751	cmt-15	5′-CAGCATCACTTTGAATTCATT	*atp8-N* 5′
K/751	atp8-u2	5′-CGCGTGGAACATGGATTAG	*atp8*
L/422	cmt-16	5′-CCGCGCTGGTCGTAATAGATA	*nad6-Sp* 5′
L/422, N/795, Sp1a/1503	nad6-u1	5′-CTTTGGGTTGGTAGTAATGGAATG	*nad6*
M/451, O/790	nad6-d1	5′-GACTACTAAGGTGAAAAGACAGG	*nad6*
M/451	cmt-17	5′-GCGAAATGCAATGCCCCAACC	*nad6-Sp* 3′
N/795	cmt-18	5′-GTTTCTGCTTACTCCTCTATGA	*nad6-N* 5′
O/790	cmt-19	5′-TGGAGCTTACCGGTTTATGG	*nad6-N* 3′
P/1229	cmt-20	5′-CTTACCGATGTTCTTTCAAGG	*cob-Sp* 5′
P/1229	cob-u2	5′-GCTTTGTCAGGTATACTACG	*cob*
Q/672	cmt-21	5′-GAATTCTCGTTTTATAGTTACAGT	*cob-N* 5′
Q/672, Sp2/1747	cob-u1	5′-CTGTCACAATCATTAATAGGAAGA	*cob*
Sp1a/1503, Sp1b/1961, Sp2/1747	atp8-d1	5′-CCGGTGTATCCTATATGTAC	*atp8*
Sp1b/1961	cmt-13	5′-CCTTGAGTCGATTCGTCCCAC	*atp6-Sp2*

cloned and sequenced. The 5′-flanking regions of the two *atp1* genes in K831B and *atp1-Sp* in K826A were identical 2.7 kb upstream from the open reading frames (ORFs), except for seven nucleotide substitutions and a 71 bp direct repeat upstream from both *atp1* genes in K831B (Figure 1). The primers flanking the direct repeat, cmt-1 and cmt-2, amplified gproducts of 321 bp and 392 bp from petaloid and fertile carrot lines, respectively (fragments A/321 and A/392 in Figure 1).

Both the *atp1-Sp* and *atp1-N1* ORFs were 1542 bp long and were predicted to encode ATP1 peptides of 513 amino acids. The *atp1-Sp* and *atp1-N1*ORFs were identical except for two silent nucleotide substitutions (772 and 1449 bp downstream from the start codon) which did not change restriction sites. The first mutation changed an arginine in N cytoplasm to a glycine in K826A, while the second mutation was silent. There was no homology between the 3′-flanking regions of *atp1-Sp* and *atp1-N1* beyond the 26th bp downstream from the stop codons. *Atp1-N2* diverged from *atp1-N1* at position 1453 within the *atp1* ORF and no stop codon was found within the cloned fragment of this chimeric ORF. Taking advantage of the fact that the three *atp1* genes had different 3′-flanking regions, PCR markers were developed for each gene. The

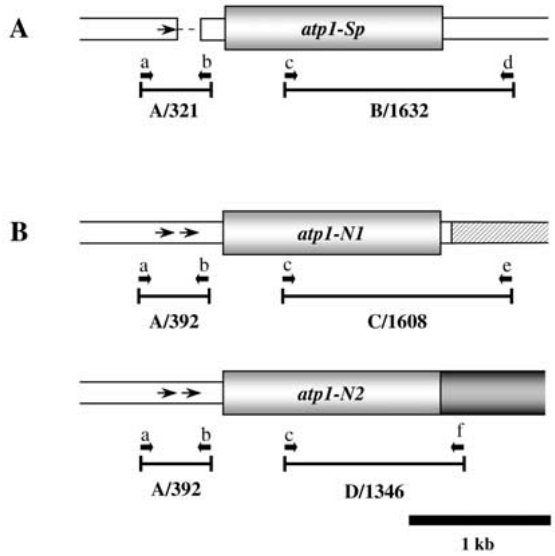

Figure 1. Structures of mtDNA *atp1* genes and flanking regions in line K826A with SpC cytoplasm (A) and line K831B with N cytoplasm (B). Open reading frames (ORFs) are indicated by large shaded boxes and intergenic regions by open boxes. Nonhomologous regions are indicated by darker shading (ORFs) or crosshatching (intergenic regions). Thin arrows indicate the sites of a 71 bp fragment that forms a direct repeat in the fertile line. The lack of this repeat upstream from *atp1-Sp* is indicated by a dotted line. Thick arrows indicate the annealing sites and orientations of the primers cmt-1 (a), cmt-2 (b), atp1-d1 (c), cmt-3 (d), cmt-4 (e) and cmt-5 (f). PCR amplification products serving as markers are indicated by bars labeled A/321, A/392, B/1632, C/1608 and D/1346. The DNA sequences of the *atp1-Sp*,*atp1-N1* and *atp1-N2* loci have been assigned GenBank Accession Nos. AF301602, AF301604 and AF301603, respectively.

primer atp1-d1, annealing within the coding region of all three *atp1* genes, amplified products specific for each *atp1* gene (fragments B/1632, C/1608 and D/1346 in Figure 1) when combined with the primers cmt-3, cmt-4 and cmt-5 specific for the 3′-flanking region of *atp1-Sp* and *atp1-N1* and the 3′-region of the *atp1-N2* ORF, respectively.

Rearrangements upstream from the atp6 *genes*

Southern hybridization indicated that both K826A and K831B contained a single *atp6* gene in the mitochondrial genome (data not shown). Both genes were cloned and sequence analysis revealed that the 5′-flanking regions of *atp6-Sp1* and *atp6-N1* diverged 40 bp upstream from the start codons (Figure 2). The 1100 bp amplification product of the primer pair cmt-6 and cmt-7 was used as a marker for the *atp6-Sp1* gene (E/1100), while the 373 bp fragment (F/373) amplified by the primers cmt-8 and atp6-u1 represented the upstream region of *atp6-N1*.

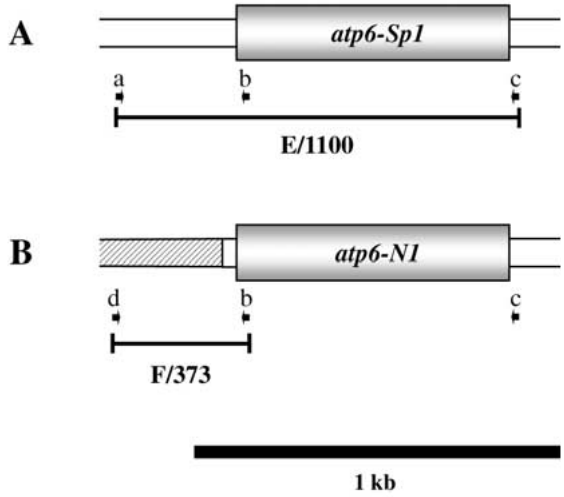

Figure 2. Configurations of *atp6* genes in K826A (A) and K831B (B). Open reading frames (ORFs) are indicated by large shaded boxes and intergenic regions by open boxes. Nonhomologous regions are indicated by crosshatching. Thick arrows labeled a-d indicate annealing sites of the primers cmt-6 (a), atp6-u1 (b), cmt-7 (c) and cmt-8 (d). PCR amplification products serving as markers are indicated by bars labeled E/1100 and F/373. The DNA sequences of the *atp6-Sp1* and *atp6-N1* loci have been assigned GenBank Accession Nos. AY007817 and AY007824, respectively.

A direct repeat downstream from the atp9 *coding region in K831B*

Complex hybridization patterns for *atp9* were observed in the petaloid and fertile lines analyzed by Szklarczyk et al. (2000) as well as in the lines K826A and K831B (data not shown). Full-length *atp9* genes were cloned from K826A and K831B and these genes were designated *atp9-Sp1* and *atp9-N1*, respectively (Figure 3). The coding regions of *atp9-Sp1* and *atp9-N1* were identical except for a single nucleotide substitution at position 229 of the ORF, which changed the stop codon TAA, observed in the fertile line, to CAA in the petaloid line. Thus, the coding region of *atp9-Sp1* was 270 bp while *atp9-N1* was 231 bp. The sequences of *atp9-Sp1* and *atp9-N1* were identical to the sequences of the *atp9* genes designated *atp9-1* and *atp9-3* from a petaloid and a fertile carrot line, respectively (Szklarczyk et al., 2000).

The 3′-flanking regions of *atp9-Sp1* and *atp9-N1* were identical for at least 176 bp downstream from the stop codon in *atp9-Sp1*, except for a 42 bp direct repeat in K831B and nucleotide substitutions at position 255

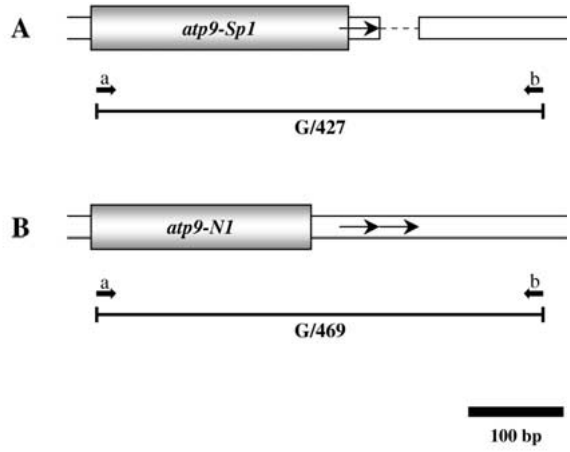

Figure 3. Structures of the full-length *atp9* genes and flanking regions in K826A (A) and K831B (B). The *atp9-N1* gene had a 42 bp direct repeat downstream from the coding region, indicated by thin arrows, resulting in amplification products of 427 and 469 bp from K826A and K831B, respectively (Bars labeled G/427 and G/469). The annealing sites of the primers atp9-d1 (a) and cmt-9 (b) are illustrated by thick arrows. A single nucleotide substitution at the site of the stop codon in *atp9-N1* resulted in extension of the *atp9-Sp1* ORF. The DNA sequences of the *atp9-Sp1* and *atp9-N1* loci have been assigned GenBank Accession Nos. AY007823 and AY007822, respectively.

and 334 relative to the start codon of *atp9-Sp1*. This direct repeat was also present in the N cytoplasm, analyzed by Szklarczyk et al. (2000). The primers atp9-d1 and cmt-9, spanning the 42 bp repeat, amplified 427 bp and 469 bp fragments from the petaloid and the fertile lines, respectively (fragments G/427 and G/469 in Figure 3).

Truncated and chimeric atp9 *genes*

A truncated *atp9* gene (*atp9-Sp2*) and a chimeric *atp9* gene (*atp9-Sp3*) from K826A and a chimeric *atp9* gene (*atp9-N2*) from K831B were located downstream from *atp8* genes as illustrated in Figure 4. The *atp9-Sp2* ORF of 147 bp and its flanking regions were identical to the *atp9-2* ORF described by Szklarczyk et al. (2000). The chimeric *atp9* ORFs, *atp9-Sp3* and *atp9-N2*, were similar and the putative coding regions were 423 bp and 417 bp, respectively. Both *atp9-Sp3* and *atp9-N2* contained a 114 bp fragment with 85% homology to the coding region of *atp6*. Downstream from each of the chimeric *atp9* ORFs small fragments with homology to the *orf25* coding region were located. Further downstream additional chimeric ORFs containing the immediate 5′-flanking region and the first 50 bp of *atp6* were present. We designated these ORFs *atp6-Sp2* and *atp6-N2*, respectively (Figure 4).

Rearrangements in the flanking regions of atp8

Southern hybridization revealed that a single copy of *atp8* (*atp8-N*) was present in K831B while three copies were detected in K826A (data not shown). The 5′-flanking region of *atp8-N* diverged from the 5′-flanking regions of the three *atp8* genes in K826A 103 bp upstream from the start codons (Figure 4). The primer pair cmt-10 and atp8-u1 amplified a 1508 bp fragment (H/1508) from K826A while no fragment was amplified from K831B. Conversely, cmt-15 and atp8-u2 amplified a 751 bp fragment (K/751) from the K831B, but no fragment was amplified from K826A.

Two configurations of *atp8* in K826A (*atp8-Sp1a* and *atp8-Sp1b*) had identical open reading frames of 651 bp but the 3′-flanking regions diverged (Figure 4). Sequence analysis of the third *atp8* copy in K826A (*atp8-Sp2*) revealed a 12 bp insert and several nucleotide substitutions in the central region and rearrangements in the 3′-region of the 711 bp ORF.The primer cmt-11 annealed upstream from the three *atp8* genes in K826A and *atp8-N* in K831B and cmt-14 annealed at the site of the insert in *atp8-Sp2*. cmt-11 and cmt-14 amplified the marker fragment J/367 specific for the 12 bp insert in *atp8-Sp2*. The 3′-flanking region of *atp8-Sp2* was extensively rearranged and the *cob* gene (*cob-Sp*) was located downstream from the *atp8-Sp2* ORF with an intergenic region of 854 bp. The truncated *atp9-Sp2* and the *nad6-Sp* gene were located downstream from the *atp8-Sp1a* gene in K826A.This configuration was identified previously by Szklarczyk et al. (2000). The 3′-flanking regions of the *atp8-N* and *atp8-Sp1b* gene were similar although the 3′-region of the putative coding regions and the immediate 3′-flanking regions differed. Amplification with the primer pair atp8-d2 and cmt-12 produced the marker fragments I/401 from K826A and I/435 from K831B. This primer pair did not distinguish *atp8-Sp1a* and *atp8-Sp1b* and did not amplify the 3′-region of *atp8-Sp2*. To evaluate variation in *atp8* configurations in different Sp lines we developed PCR markers specific for the 3′-flanking regions of each *atp8* gene. The marker fragment Sp1a/1503 specific for the *atp8-Sp1a* configuration was amplified with the primer atp8-d1 in combination with nad6-u1. Primer atp8-d1 combined with cob-u1 amplified the 3′-flanking region of *atp8-Sp2* (fragment Sp2/1747), while atp8-d1 and cmt-13 amplified the 3′-flanking region of *atp8-Sp1b*

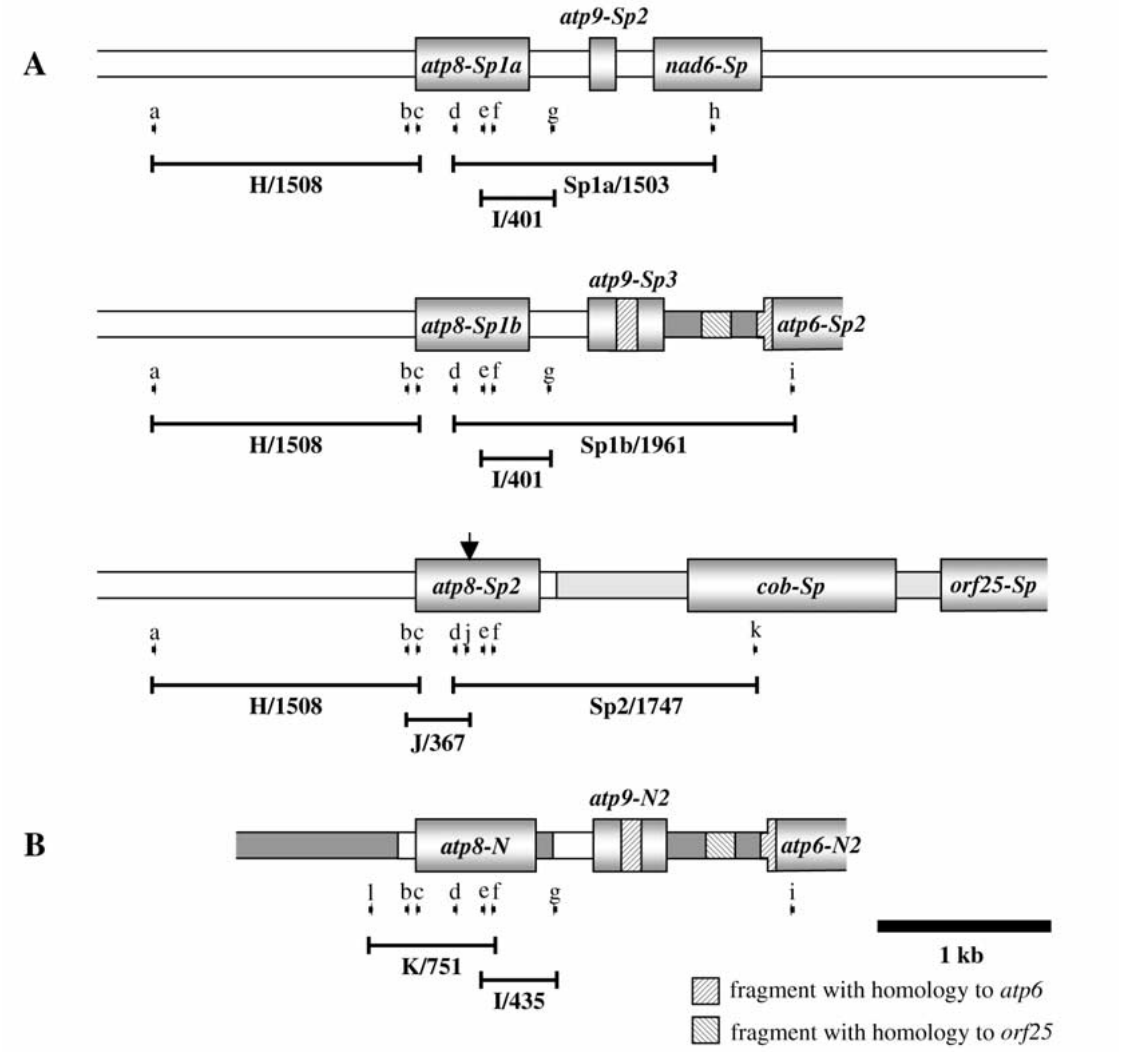

Figure 4. Configurations of *atp8* loci in K826A (A) and K831B (B). Three copies of *atp8* were present in the K826A. The 3-flanking region of *atp8-N* was similar to the 3′-flanking region of *atp8-Sp1b*. Nonhomologous intergenic regions are indicated by different cross hatching patterns. Joined fragments in chimeric coding regions are indicated by different shadings. Thick arrows indicate annealing sites and orientations of the primers: cmt-10 (a), cmt-11 (b), atp8-u1 (c), atp8-d1 (d), atp8-d2 (e), atp8-u2 (f), cmt-12 (g), nad6-u1 (h), cmt-13 (i), cmt-14 (j), cob-u1 (k) and cmt-15 (l). Bars labeled H/1508, I/401, I/435, J/367 and K/751 indicate marker fragments that distinguish the mtDNA from K826A and K831B. Sp1a/1503, Sp1b/1961 and Sp2/1747 differentiate the three *atp8* configurations in K826A. The vertical arrowhead indicates the site of the unique 12 bp insert in *atp8-Sp2*. The DNA sequences of the *atp8-Sp1a*, *atp8-Sp1b*, *atp8-Sp2*, and *atp8-N* loci have been assigned GenBank Accession Nos. AY007819, AY007820, AY007821 and AY007818, respectively.

(Sp1b/1961). The latter primer pair also amplified a 2010 bp fragment from the *atp8-N* configuration in K831B.

Rearrangements both upstream and downstream from the nad6 *genes*

The *nad6-Sp* gene was located downstream from *atp8-Sp1a* and *atp9-Sp2*, as indicated in Figure 4. The alignment of the *nad6* gene in K831B (*nad6-N*) and *nad6-Sp* sequences revealed identical coding regions of 618 bp except for two silent nucleotide substitutions. An additional point mutation was identified 52 bp upstream from the start codons and the sequences diverged 151 bp upstream from the ORFs (Figure 5). The primer cmt-16 was designed to anneal upstream from *nad6-Sp* at the site of this point mutation and the primers cmt-16 and nad6-u1 amplified a 422 bp fragment (L/422) from K826A but not from K831B. The

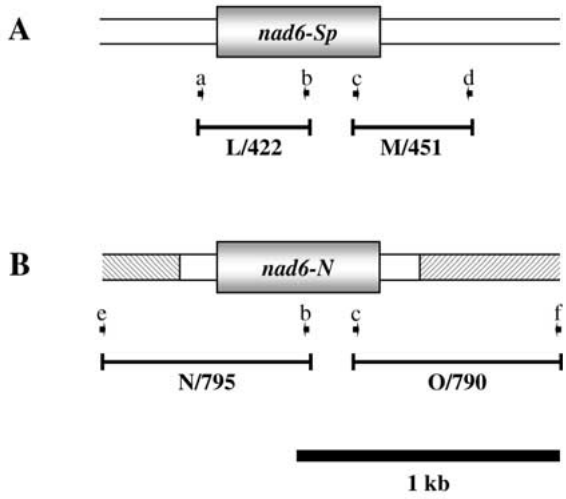

Figure 5. Configurations of *nad6* loci in K826A (A) and K831B (B). Crosshatching indicates nonhomologous regions. Thick arrows indicate annealing sites of the primers cmt-16 (a), nad6-u1 (b), nad6-d1 (c) cmt-17 (d) cmt-18 (e) and cmt-19 (f). The marker fragments, L/422, M/451, N/795 and O/790 are indicated as bars. The DNA sequences of the *nad6-Sp* and *nad6-N* loci have been assigned GenBank Accession Nos. AY007819 and AY007818, respectively.

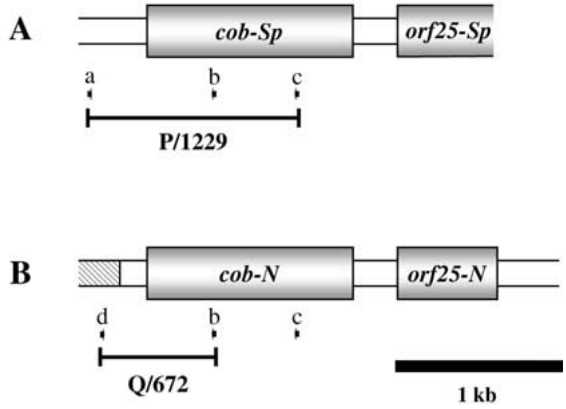

Figure 6. Configurations of *cob-Sp* (A) in K826A and *cob-N* (B) in K831B. The crosshatched region upstream from *cob-N* had no homology to the 5′-flanking region of *cob-Sp*. Thick arrows indicate annealing sites of the primers cmt-20 (a), cob-u1 (b), cob-u2 (c) and cmt-21 (d). The marker fragments P/1229 and Q/672 are indicated as bars. The DNA sequences of the *cob-Sp* and *cob-N* loci have been assigned GenBank Accession Nos. AY007821 and AY007816, respectively.

primer pair cmt-18 and nad6-u1 specifically amplified a 795 bp fragment (N/795) from K831B.

Nad6-N and *nad6-Sp* diverged 286 bp downstream from the stop codons, and primers specific for each 3′-flanking region were designed (Figure 5). The primers nad6-d1 and cmt-17 amplified a 451 bp fragment (M/451) from K826A and nad6-d1 together with cmt-19 amplified a 790 bp fragment (O/790) from K831B.

Divergence upstream from the cob *open reading frames in K826A and K831B*

Southern hybridization revealed that the *cob* genes were present as single copy genes in both K826A and K831B (data not shown). The ORFs of *cob-N* and *cob-Sp* coding regions were 1197 bp and identical, except for four nucleotide substitutions of which one potentially alters a Leu residue in K831B to an Ile residue in K826A. The 5′-flanking regions of *cob-N* and *cob-Sp* diverged 158 bp upstream from the start codons (Figure 6). The primer cmt-20, annealing to the 5′-flanking region of *cob-Sp* upstream from the breakpoint, and the primer cob-u2, amplified a 1229 bp fragment (P/1229) from K826A while no amplification with this primer pair was observed from K831B. The primer, cmt-21, specific for the upstream region of *cob-N*, combined with cob-u1 amplified a 672 bp fragment (Q/672) from K831B.

Association of markers to type of cytoplasm in different nuclear backgrounds

General use of mitochondria-specific markers depends upon consistent differences between sterile and fertile cytoplasms and uniformity in marker fragment generation from a cytoplasm in diverse nuclear backgrounds. We tested our markers on a diverse selection of USDA breeding lines, commercial breeding lines and the commercial hybrid ‘Savory’ (Table 1).

The markers representing absence or presence of the 71 bp repeat upstream from the *atp1* genes (fragments A/321 and A/392) consistently distinguished SpC and SpW lines and the hybrid ‘Savory’ from all maintainer lines analyzed. The A/321 fragment was present in all Sp material evaluated in this study while the A/392 fragment always occurred in N lines. Also the markers representing the 3′-flanking regions of *atp1-Sp* (B/1632) and *atp1-N1* (C/1608) were consistently associated with Sp and N cytoplasms, respectively. The marker for the chimeric gene, *atp1-N2* (D/1346), was present in all but one (B3475B) of the N lines analyzed and absent from all but one Sp line (K803A).

The configuration of the 5′-flanking regions of the *atp6* genes was conserved among the N lines tested and the marker for *atp6-N1* (F/373) was amplified

from all N lines while the *atp6-Sp1* specific marker (E/1100) was present in all Sp material analyzed. The markers representing presence or absence of a direct repeat in the *atp9* region (G/427 and G/469) distinguished Sp material from N lines. However, mutations resulting in loss of the Sp-specific fragment G/427 had occurred in both an SpC ('Savory') and an SpW cytoplasm (B2566A).

Fragment H/1508 representing the 5′-region of the *atp8-Sp* genes was amplified from all Sp but none of the N lines analyzed, while K/751 corresponding to the 5′-region of *atp8-N* was restricted to N lines. Fragment I/401 was amplified from all Sp materials except K803A, while marker fragments of either 435 bp or 214 bp were amplified from N lines (Table 1; Figure 4). Inconsistent results were obtained for J/367 specific for the unique insert in coding region of *atp8-Sp2*. J/367 was present in three of seven SpC materials and one of two SpW lines analyzed. Deviation within Sp lines of the markers specific for the 3′-configurations of *atp8-Sp1a*, *atp8-Sp1b* and *atp8-Sp2* was also observed. For example, the markers Sp1a/1503 and Sp1b/1961 were absent from the SpC line K803A. Southern hybridization indicated that *atp8-Sp1a* and the *atp8-Sp1b* had been lost from the mitochondrial genome of K803A and cloning and sequencing of the 5′-flanking region of the *nad6* gene from K803A revealed rearrangements upstream from the annealing site of cmt-16 (data not shown).

In spite of rearrangements upstream from *nad6-Sp* (loss of *atp8-Sp1a* from K803A) and *cob-Sp* (loss of *atp8-Sp2* from B2566A) the markers L/422 and P/1229 specific for the 5′-flanking regions of *nad6-Sp* and *cob-Sp* were present in all Sp material analyzed and absent from all N lines analyzed. The marker Q/672 anchored in the 5′-flanking region of *cob-N* was associated with the N cytoplasms with two exceptions; absence of Q/672 from the N lines B3475B and K803B.

The marker M/451 anchored in the 3′-flanking region of *nad6-Sp* was present in all Sp materials but was not confined to Sp cytoplasms as M/451 was amplified from the fertile lines, B3475B and K803B. Inconsistent results were also obtained with the markers for the 5′- and 3′-flanking regions of *nad6-N* (N/795 and O/790), as both N/795 and O/790 were absent from the N lines B3475B and K803B and a faint O/790 band was observed in the Sp line B493A.

Discussion

In this study we designed 17 primer pairs that amplify mitochondria-specific PCR markers that distinguish petaloid CMS cytoplasms (Sp) from fertility-inducing cytoplasms (N). Three additional primer pairs distinguished different configurations of the *atp8* genes in Sp cytoplasms. Of six previously published primer pairs amplifying carrot mitochondrial DNA only two primer pairs amplified marker fragments that were consistently associated with either Sp or N cytoplasms (Nakajima et al., 1999). Of the 17 primer pairs reported here eleven amplified marker fragments that distinguished all Sp from N cytoplasms analyzed. These markers are being used to identify the type of cytoplasm and test seed purity in breeding programs, to select cybrids after protoplast fusion, and to study basic diversity in the genus *Daucus*. The markers reported here are anchored within or immediately adjacent to the coding regions of conserved mitochondrial genes and reflect rearrangements of the mitochondrial genomes.

The markers located in the 5′- and 3′-regions of *atp1-Sp* (A/321 and B/1632), the 5′-region of *atp6-Sp1* (E/1100), the 5′-regions of the *atp8 Sp* genes (H/1508), the 5′-region of *nad6-Sp* (L/422) and the 5′-region of *cob-Sp* (P/1229) from the Sp cytoplasms were strictly associated with the Sp material analyzed. Markers restricted to and amplified from all N lines analyzed were located in the 5′- and 3′-regions of the conserved *atp1-N1* coding region (A/392 and C/1608), the 5′-region of *atp6-N1* (F/373), the 5′-region of *atp8* (K/751) and the 3′-region of the full-length *atp9-N1* gene (G/469). In addition, the 3′-flanking regions of *atp8* from all N lines were amplified with a single primer pair, resulting in the N specific markers I/435 or I/214. The variation among N cytoplasm was reflected by absence of the markers D/1346, N/795, O/790 and Q/672 from some lines.

Rearrangements in the *atp8* and *atp9-Sp1* loci in the Sp cytoplasms resulted in inconsistent marker amplification. For example, presence of the marker J/367 specific for the unique insert in the coding region of *atp8-Sp2* in both SpC and SpW lines suggested that this insert was present in the original SpC and SpW cytoplasms and subsequently lost in some of the breeding material analyzed. Also the variation in the markers specific for the 3′-configurations of *atp8-Sp* genes indicated that the duplicated *atp8* genes were rearranged since these cytoplasms were combined with the nuclear backgrounds of cultivated carrot.

Some of our markers (fragments D/1346, G/427, I/401, J/367, and O/790) varied among Sp material, but we found no consistent differences between lines with SpC and SpW cytoplasm. The marker STS2 was reported by Nakajima et al. (1999) to be present in SpW cytoplasms but not in SpC cytoplasms. We analyzed SpC lines and SpW lines with different nuclear backgrounds for presence of this marker and found that the STS2 marker was present in 11 of 15 SpC lines and 6 of 7 of the SpW lines analyzed (data not shown). We suggest that the observed variation among Sp lines reflects the dynamic nature of the mitochondrial genomes and possibly the effect of different nuclear backgrounds, rather than evidence of independent origins among Sp cytoplasms. The fact that SpC and SpW both originated from single plants in 1953 and 1970, respectively, attests to this dynamic nature.

The N specific marker O/790 was present as a faint band in one Sp line (B493A). Mitochondrial DNA molecules or gene configurations can be present at very low copy number (Bellaoui et al., 1998; Small et al., 1987) and the observed faint band may be amplified from low level of mtDNA configurations identical or similar to the configurations in the N cytoplasms.

The variation among the different types of N cytoplasms in cultivated carrot and the evidence for frequent rearrangements of the mitochondrial genomes, illustrates the importance of having several markers that distinguish Sp cytoplasms from N cytoplasms. Furthermore, heteroplasmy or the presence of substoichiometric amounts of variant gene configurations, and also the transfer of genes from the mitochondria to the nucleus (Lin et al., 1999; Martin & Herrmann, 1998) increase the importance of having several markers to predict male fertility or male sterility in seed lots or individual plants.

The role of the described differences between the Sp and N mitochondrial genomes in flower development remains to be elucidated. A complete *atp1* gene (*atp1-N1*) and a chimeric *atp1* gene (*atp1-N2*) were identified in several fertile carrot lines, while only one *atp1* gene (*atp1-Sp*) was identified in petaloid lines. The presence of a transcribed chimeric *atp1* gene in a petaloid tobacco cybrid (Bergman et al., 1995, Glimelius et al., 1995) suggested a possible involvement of an aberrant *atp1* gene in petaloidy. As the chimeric *atp1* gene from carrot was found in N cytoplasms, it is unlikely that this gene influences flower development. A direct repeat was present upstream from both *atp1* copies from the maintainer line K831B and also single nucleotide substitutions were identified in the 5′-flanking region of *atp1-Sp*, when compared to *atp1-N1* and *atp1-N2*. Two transcripts with homology to *atp1* were observed in petaloid carrot by Scheike et al. (1992) and Szklarczyk (1997). The longer of the two transcripts was either undetected or present in very low amounts in N cytoplasms, while abundant in the Sp cytoplasms analyzed. We hypothesize that the rearrangements in the 5′-flanking regions of the *atp1* genes affect either transcription initiation or processing of the *atp1* primary transcript. Whether the longer transcript with homology to *atp1* is involved in male sterility in carrot remains to be investigated. Nevertheless, the structural differences in the regions adjacent to the three *atp1* genes have been useful for the development of markers specific for fertile and petaloid cytoplasms.

A gene construct containing the unedited *atp9* gene from wheat encoded an ATP9 peptide with a few internal amino acid substitutions and additional 6 amino acids at the carboxy terminus (Hernould et al., 1993). When expressed in tobacco, this gene caused male sterility, while the corresponding transgene derived from the cDNA did not affect flower development. The *atp9-Sp1* gene from petaloid carrot had a point mutation at the site of the stop codon of the *atp9-N1* from K831B. This mutation caused an extension of the ORF of *atp9-Sp* in comparison to the ORF of *atp9-N1*. The primary transcripts of most mitochondrial genes are edited and in some cases, stop codons are introduced by RNA editing forming a conserved coding region (reviewed by Marchfelder et al., 1998 and Mulligan et al., 1999). Thus, the transcript of either *atp9-Sp1* or *atp9-N1* may be edited to obtain identical coding regions and ATP9 peptides. If no editing occurs, the ATP9 peptide from the petaloid line would have a C-terminal extension of 13 amino acids in comparison to ATP9 from the fertile line. Lack of general or tissue specific editing of the *atp9* gene from petaloid carrot could affect flower development. Analysis of expression and editing of *atp9* genes and their derivatives will help establish their role in carrot CMS. Szklarczyk et al. (2000) found variation in the expression patterns of *atp9* homologous transcripts from petaloid, partially restored petaloids and fertile carrot plants, indicating a possible relationship with CMS. Further studies are needed to determine which of the *atp9* genes from the Sp cytoplasms are differentially expressed.

Comparison of the 5′-flanking regions of the genes *atp6*, *cob* and *nad6* from Sp and N cytoplasms re-

vealed rearrangements in these regions. Also the 3′-flanking regions of the *nad6* genes differed. Unless the rearrangements in the flanking regions of *atp6*, *cob* and *nad6* affect transcription, editing of the primary transcript, RNA stability or translation, the differences in configuration of these genes are not expected to affect flower development, as the coding regions are almost identical.

Three copies of *atp8* from the petaloid line K826A were cloned and sequenced. One of these copies (*atp8-Sp2*) had a unique insert in the coding region. Only this copy could be detected in the petaloid line K803A, strongly suggesting that a functional ATP8 peptide is translated from an *atp8-Sp2* transcript. Nakajima et al. (1999) reported correlation between the petaloid phenotype in carrot cybrids and presence of an STS-marker specific for the 5′-flanking region of an *atp8* gene. Presence of a mutated *atp8* gene in both SpC and SpW cytoplasms suggested that the insertion of four extra aminoacids in ATP8 could be associated with expression of petaloidy. However, we could not amplify the insert-specific PCR marker J/367 from all petaloid carrot lines and unless this *atp8* copy is still expressed but mutated in a way that makes it undetectable with the insert specific primer pair tested, this aberrant *atp8* gene in carrot is not likely to be responsible for petaloid CMS.

We observed an inframe ATG codon upstream from the conserved translation initiation site of all three *atp8-Sp* copies. The marker H/1508 specific for this 5′-configuration of *atp8* was present in all Sp material analyzed but none of the N lines. This 5′-configuration of the *atp8-Sp* genes may result in expression of ATP8 peptides with a 5′-extension. The *atp8* coding regions from different carrot cytoplasms are highly variable in the 3′-ends and both *atp8-N* and the *atp8-Sp* coding regions are significantly longer than the edited versions of *atp8* from other species, for example wheat (*orf156*, Gualberto et al., 1991). We speculate that stop codons are introduced by editing of the primary *atp8* transcripts from carrot to form functional ATP8 peptides. Analysis of editing patterns of the *atp8* transcripts from male sterile and fertile carrot lines will reveal differences in the *atp8* coding regions in mature mRNAs. Presence of aberrant ATP8 peptides may provide a possible correlation with expression of CMS.

In several species specific mitochondrial genes have been identified as the cause of CMS (reviewed in Schnable & Wise, 1998). Numerous changes have been observed in the mitochondrial genome of petaloid carrots, relative to their male fertile counterparts with N cytoplasm and petaloidy may have a multigenic basis. Petaloid tobacco stamens can produce functional pollen (Bergman et al., 1995), suggesting that petaloidy and male-sterility could be caused by different mitochondrial genes. We have identified several rearrangements in the vicinity of mitochondrial genes from the petaloidy and male-sterility inducing cytoplasms in carrot compared to different N cytoplasms. Further investigations are needed to determine if any of these rearrangements are directly involved in either petaloidy or lack of development in carrots carrying Sp cytoplasm.

Acknowledgements

Laboratory facilities and supervision for preparation of the mtDNA libraries was kindly provided by Dr. Wolfgang Schuster.

References

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller & D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

Bach, I.C., O.M. Madsen & A Olesen, 1997. Production of a mapping population by protoplast fusion for the localisation of CMS-genes in carrot. J Appl Genet 38A: 178–185.

Banga O., J. Petiet & J.L. van Bennekom, 1964. Genetic analysis of male-sterility in carrot. Euphytica 13: 75–93.

Bellaoui M., A. Martin-Canadell, G. Pelletier & F. Budar F, 1998. Low copy-number molecules are produced by recombination, actively maintained and can be amplified in the mitochondrial genome of *Brassicaceae*: relationship to reversion of the male sterile phenotype in some cybrids. Mol Gen Genet 257: 177–185.

Bergman P. W. Kofer, G. Håkansson & K. Glimelius, 1995. A chimeric and truncated mitochondrial *atpA* gene is transcribed in alloplasmic cytoplasmic male-sterile tobacco with *Nicotiana bigelovii* mitochondria. Theor Appl Genet 91: 603–610.

Bowes, C.E. & D.J. Wolyn, 1998. Phylogenetic relationships among fertile and petaloid male sterile accessions of carrot, *Daucus carota* L. Theor Appl Genet 96: 928–932.

Glimelius, K., M. Hernould & P. Bergman, 1995. Mitochondrial regulation of petal and stamen development in cytoplasmic male sterile cultivars of *Nicotiana tabacum*. In: M. Terzi, R. Cella & A. Falavigna (Eds.), Current Issues in Plant Molecular and Cellular Biology, pp. 219–224. Kluwer Academic Publishers.

Gray, M.W., B.F. Lang, R. Cedergren, G.B. Golding, C. Lemieux, D. Sankoff, M. Turmel, N. Brossard, E. Delage, T.G. Littlejohn, I. Plante, P. Rioux, D. Saint-Louis, Y. Zhu & G. Burger, 1998. Genome structure and gene content in protist mitochondrial DNA. Nucleic Acids Res 26: 865–878.

Gualberto, J.M., G. Bonnard, L. Lamattina & J.M. Grienenberger, 1991. Expression of the wheat mitochondrial *nad3-rps12* transcription unit: Correlation between editing and mRNA maturation. Plant Cell 3: 1109–1120.

Hernould, M., S. Suharsono, S. Litvak, A. Araya & A. Mouras, 1993. Male-sterility induction in transgenic tobacco plants with an unedited *atp9* mitochondrial gene from wheat. Proc Natl Acad Sci USA 90: 2370–2374.

Lin, X.Y., S.S. Kaul, S. Rounsley, T.P. Shea, M.I. Benito, C.D. Town, C.Y. Fujii, T. Mason, C.L. Bowman, M. Barnstead, T.V. Feldblyum, C.R. Buell, K.A. Ketchum, J. Lee, C.M. Ronning, H.L. Koo, K.S. Moffat, L.A. Cronin, M. Shen, G. Pai, S. Van Aken, L. Umayam, L.J. Tallon, J.E. Gill, M.D. Adams, A.J. Carrera, T.H. Creasy, H.M. Goodman, C.R. Somerville, G.P. Copenhaver, D. Preuss, W.C. Nierman, O. White, J.A. Eisen, S.L. Salzberg, C.M. Fraser & J.C. Venter, 1999. Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. Nature 402: 761–768.

Linke, B., T. Nothnagel & T. Borner, 1999. Morphological characterization of modified flower morphology of three novel alloplasmic male sterile carrot sources. Plant Breeding 118: 543–548.

Marchfelder, A., S. Binder, A. Brennicke & W. Knoop, 1998. RNA editing by base conversion in plant organellar DNA. In: H. Grosjean & R. Benne (Eds.), Modification and Editing of RNA, pp. 307–323. ASM Press, Washington D.C.

Martin, W. & R.G. Herrmann, 1998. Gene transfer from organelles to the nucleus: How much, what happens, and why? Plant Physiol 118: 9–17.

McCollum, G.D., 1966. Occurrence of petaloid stamens in wild carrot (*Daucus carota*) from Sweden. Economic Botany 20: 361–367.

Morelock, T.E., P.W. Simon & C.E. Peterson, 1996. Wisconsin Wild: Another petaloid male-sterile cytoplasm for carrot. HortSci 31: 887–888.

Mulligan, R.M., M.A. Williams & M.T. Shanahan, 1999. RNA editing site recognition in higher plant mitochondria. J Heredity 90: 338–344.

Murray, M.G. & W.F. Thompson, 1980. Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8: 4321–4325.

Nakajima Y., T. Yamamoto, T. Muranaka & K. Oeda, 1999. Genetic variation of petaloid male-sterile cytoplasm of carrot revealed by sequence tagged sites (STSs). Theor Appl Genet 99: 837–843.

Nothnagel, T., P. Straka & B. Linke, 2000. Male sterility in populations of *Daucus* and the development of alloplasmic male-sterile lines of carrot. Plant Breeding 119: 145–152.

Pingitore, M., B. Matthews & J.P. Bottino, 1989. Analysis of the mitochondrial genome of *Daucus carota* with male sterile and male fertile cytoplasm. J Heredity 80: 143–145.

Ronfort, J., P. Saumitou-Laprade & J. Cuguen, 1995. Mitochondrial DNA diversity and male sterility in natural populations of *Daucus carota* ssp *carota*. Theor Appl Genet 91: 150–159.

Sambrook, J., E. Fritsch & T. Maniatis, 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Scheike, R., E. Gerold, A. Brennicke, M. Mehring-Lemper & G. Wricke, 1992. Unique patterns of mitochondrial genes, transcripts and proteins in different male-sterile cytoplasma of *Daucus carota*. Theor Appl Genet 83: 419–427.

Schnable, P.S. & R.P. Wise, 1998. The molecular basis of cytoplasmic male sterility and fertility restoration. Trends in Plant Science 3: 175–180.

Schuster, W., R. Hiesel, B. Wissinger, W. Schobel & A. Brennicke, 1988. Isolation and analysis of plant mitochondria and their genomes. In: C. Shaw (Ed.), Plant Molecular Biology. A Practical Approach, pp. 79–102. IRL Press. Oxford. Washington DC.

Small, I.D., P.G. Isaac & C.J. Leaver, 1987. Stoichiometric differences in DNA molecules containing the *atpA* gene suggests mechanisms for the generation of mitochondrial diversity in maize. EMBO J 6: 865–869.

Steinborn, R.A., A. Weihe & T. Börner, 1992. Mitochondrial genome diversity within a cultivar of *Daucus carota* (ssp. *sativus*) revealed by restriction fragment analysis of single plants. Plant Breeding 109: 75–77.

Szklarczyk, M., 1997. Unique features of carrot mtDNAs from CMS and maintainer lines. J Appl Genet 38A: 42–56.

Szklarczyk, M., M. Oczkowski, H. Augustyniak, T. Börner, B. Linke & B. Michalik, 2000. Organisation and expression of mitochondrial *atp9* genes from CMS and fertile carrots. Theor Appl Genet 100: 263–270.

Thompson, D.J., 1961. Studies on the inheritance of male-sterility in the carrot, *Daucus carota* L. var. *sativa.* Proc Amer Soc Hort Sci 78: 332–338.

Unseld, M., J.R. Marienfeld, P. Brandt & A. Brennicke, 1997. The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nature Genet 15: 57–61.

Vivek, B.S., Q.A. Ngo & P.W. Simon, 1999. Evidence for maternal inheritance of the chloroplast genome in cultivated carrot (*Daucus carota* L. ssp. *sativus*). Theor Appl Genet 98: 669–672.

Vivek, B.S. & P.W. Simon, 1999. Phylogeny and relationships in *Daucus* based on restriction fragment length polymorphisms (RFLPs) of the chloroplast and mitochondrial DNA. Euphytica 105: 183–189.

Welch, J.E. & E.L. Grimball, 1947. Male sterility in carrot. Science 106: 594.